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**The H<sup>+</sup>-linked monocarboxylate transporter (*MCT1/SLC16A1*): a potential  
therapeutic target for high-risk neuroblastoma**

Jun Fang, Quintin J. Quinones, Trevor L. Holman, Michael J. Morowitz, Qun Wang,

Huaqing Zhao, Frank Sivo, John M. Maris, Miriam L. Wahl

*Department of Pathology, Duke University Medical Center, Durham, NC (J.F., Q.J.Q., T.L.H., F.S., M.L.W.) and Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine and Abramson Family Cancer Research Institute, Philadelphia, PA (M.J.M., Q.W., H.Z., J.M.M.).*

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- a) Running Title: MCT1 as a therapeutic target in neuroblastoma
- b) Corresponding author: Miriam L. Wahl, Ph.D., Department of Pathology, Box 3712, Duke University Medical Center, Durham, North Carolina 27710.  
Phone: (919) 668-1619, Fax: (919) 668-1218, Email:  
[Miriam.Wahl@duke.edu](mailto:Miriam.Wahl@duke.edu)
- c) Number of text pages: 17  
Number of tables: 2  
Number of figures: 8  
Number of words in Abstract: 211  
Number of words in the Introduction: 305  
Number of words in the Discussion: 1467  
Number of references: 40

Abbreviations: CNCn,  $\alpha$ -cyano-4-hydroxy cinnamate; C.SNARF-1AM, carboxyl semi-naphthyl rhodofluor-1 acetoxymethyl ester; FBS, fetal bovine serum; GADPH, glyceraldehyde phosphate dehydrogenase; *MCT1/SLC16A1*; H<sup>+</sup>-linked monocarboxylate transporter, isoform 1; *LON*, lonidamine (TH-070; 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid); MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; *MYCN* transcription factor mycN.

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## ABSTRACT

Neuroblastomas produce high amounts of lactic acid and upregulate the H<sup>+</sup>-linked monocarboxylate transporter, isoform 1 (*MCT1/SLC16A1*). We found elevated MCT1 mRNA levels in fresh neuroblastoma biopsy samples, which correlated positively with risk of fatal disease and amplification of the “protooncogenic” transcription factor MYCN. We further investigated MCT as a potential therapeutic target *in vitro*. The neuroblastoma cell lines evaluated were Sk-N-SH, CHP134, IMR32, and NGP. All lines exhibited decreased intracellular pH at low tumor-like extracellular pH. Intracellular pH was lowered further by lonidamine or exogenous lactate. Immediate, early lowering of intracellular pH with lonidamine or lactate at extracellular pH 6.5 correlated positively with diminished cell viability within 48 h. These findings indicate that MCT1 is a potential therapeutic target and that neuroblastoma therapy may be enhanced by therapeutic strategies to inhibit or overwhelm MCT. Additional experiments indicated that the mechanism of cell death by lonidamine or exogenous lactate is similar to that obtained using  $\alpha$ -cyano-4-OH-cinnamate (CNCn), a well-established MCT inhibitor. Because lactate production is also high in melanoma and many other tumor types, MCT inhibitors may have broad application in cancer treatment. Such treatment would have selectivity by virtue of the acidic milieu surrounding tumors, since MCT is increasingly active as extracellular pH decreases below 7.0 and lactic acid production increases.

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## Introduction

Neuroblastomas are childhood malignancies postulated to originate from precursor cells of the postganglionic sympathetic nervous system (Brodeur, 2003). The disease is remarkable for its clinical heterogeneity, with spontaneous regression being common in infants diagnosed before the age of 12 months, but relentless malignant progression being common in children diagnosed after their first birthday. This latter category of “high-risk” patients represents 50% of all cases. Moreover, mortality in this subset remains greater than 60% despite aggressive chemoradiotherapy (Brodeur, 2002; Matthay et al., 1999). Clearly, new treatment approaches are needed.

High lactic acid production is a common feature of many solid-tumor cells (Brizel et al., 2001; Skoyum et al., 1997; Walenta et al., 1997). Recently, the transporters responsible for export of lactic acid have been characterized in detail (Halestrap and Meredith, 2004). Further, we have shown that MCT isoform 1 (MCT1) and MCT isoform 4 (MCT4) both have elevated activity in human melanoma cells in response to low extracellular pH (Wahl et al., 2002). This suggests that the level and/or affinity of proton export transporters may be increased in cancer cells and that these transporters function to prevent lethal intracellular pH decreases as tumors grow and become more acidotic. In a recent microarray screen, we noted differential expression of *MCT1/SLC16A1* mRNA, but not of any other SLC16A family members in relation to tumor phenotype (Wang et al., 2006). We therefore hypothesized that expression of one or more of the monocarboxylate transport proteins would be associated with more malignant subsets of neuroblastoma because these are the tumors most likely to be subjected to great pH stress. We further suggest that pharmacologic manipulation of these transport proteins is feasible and that the inhibition of such proteins may define a new class of novel therapeutics for neuroblastoma, and perhaps other neural-crest derived malignancies such as melanoma (Balazs et al., 2001; Wahl et al., 2002).

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## Materials and Methods

**Cell lines and primary tumors:** The neuroblastoma cell lines IMR32, Sk-N-SH, and NGP were obtained from the American Tissue Culture Collection (Manassas, VA). CHP134 cells were isolated from a patient at the Children's Hospital of Philadelphia. Rho-negative SY5Y neuroblastoma cells were generously provided by Dr. W. Davis Parker, Jr. (U. of Virginia School of Medicine, Charlottesville, VA).

Cells were grown in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 26 mM sodium bicarbonate. Media was supplemented with 2 mM glutamine and 1% penicillin/streptomycin. Cells were maintained in a humidified 37°C incubator at 5% CO<sub>2</sub> for a pH of 7.3, or 15 mM sodium bicarbonate, 17% CO<sub>2</sub> to lower extracellular pH to 6.5. All of these cell lines were maintained in logarithmic growth at 37°C as monolayers in 75 cm<sup>2</sup> flasks.

Eighty-three primary neuroblastomas were obtained at diagnosis from children enrolled in Children's Oncology Group (COG) Biology studies. Patient clinical characteristics and risk group were determined using standard COG algorithms as described (Maris, 2005). Biopsy samples were snap frozen in liquid nitrogen and a diagnosis of neuroblastoma was confirmed by central pathologic review. *MYCN* gene copy number status was determined by fluorescence hybridization *in situ*; DNA index was determined by flow cytometry following cell dissociation (Look et al., 1991; Matthay et al., 1999). We estimated the percent of tumor cell content in pilot sections before nucleic acid extraction. Only samples with a greater than 70% neuroblastoma cell content were included in this study.

We isolated RNA from exponentially growing cell lines or from 50-100 mg of snap-frozen tumor samples using TRIzol reagent (Invitrogen, Carlsbad, CA). All RNA samples were subjected to DNase I (Ambion, Houston, TX) digestion to eliminate contaminating

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genomic DNA, then purified with QIAGEN RNeasy Mini Kit (Valencia, CA). RNA quality was checked by spectrophotometry, 1% agarose gel, as well as by microfluidics-based electrophoretic analysis (Agilent 2100 Bioanalyzer). The Children's Hospital of Philadelphia Institutional Review Board approved this study.

**Reagents:** We obtained the fluorochrome Carboxy SNARF-1 acetoxymethyl ester (C.SNARF-1AM) and the detergent Pluronic F127 from Molecular Probes (Eugene, OR). Matrigel was purchased from BD Biosciences (Bedford, MA). The MTS kit was obtained from Promega (Madison, WI). All PCR reagents except Tri Reagent were from Invitrogen (Carlsbad, CA). We obtained Tri Reagent and all other reagents, including Ionidamine and  $\alpha$ -cyano-4-hydroxy cinnamate (CNCn), from Sigma Chemical Co. (St. Louis, MO). Ionidamine was diluted into media from a 16 mM stock solution of DMSO. DMSO and other vehicle controls were negative with respect to effects on intracellular pH and viability.

**Western Blot Materials:** Primary antibodies to MCT1 (C-20) and *MycN* (H-50) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody IR dye conjugates donkey anti-goat IR Dye 800 nm and goat anti-rabbit Alexa Fluoro 680 nm were obtained from Rockland (Gilbertsville, PA) and Molecular Probes (Carlsbad, CA) respectively. Cell Death Detection ELISA Plus 10x was obtained from Roche Applied Science (Indianapolis, IN). MYCN siRNA was obtained from Ambion (Austin, TX) siRNA ID# 114526 Sense strand 5' GGAGCAUGUUUUGUAUACAtt 3' Antisense Strand 5' UGUUAUACAAAACAUGCUCctc 3', Silencer Negative Control #1 siRNA was also obtained from Ambion. Lipofectamine 2000 was obtained from Invitrogen Corporation (Carlsbad, CA).

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### **Western Blotting Methods:**

Neuroblastoma cells were solubilized in aqueous buffer containing 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris, and 20% glycerol at pH 6.8, scraped from T-25 cell culture flasks, and placed in microfuge tubes. Cells were pulsed for 5 seconds with a probe sonicator, vortexed for 10 s, and centrifuged at 1000xg for 2 min. The protein levels of the samples were measured by BioRad BCA assay. Samples were all standardized to 0.5 mg/mL protein by dilution with gel loading buffer containing 2% SDS, 62.5mM Tris, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue at pH 6.8. Samples were heated in boiling water for 5 min and then centrifuged at 1000 x g for 5 minutes. Each sample (20  $\mu$ L) was loaded into each well of a 4-15% polyacrylamide Tris HCl Bio Rad Ready Gel for a total of 10  $\mu$ g of protein per lane. PAGE was performed at 180V constant voltage in Tris/Glycine/SDS running buffer 25 mM/192 mM/0.1% pH 8.3 for 1 h. Protein was transferred to Amersham Biosciences Hybond-C Extra Nitrocellulose 45  $\mu$ m for 30 min at 15V constant voltage on a BioRad Transblot Semi-dry transfer cell in Tris/Glycine buffer 25 mM/192 mM containing 10% methanol at pH 8.3. Membranes were blocked in Rockland blocking buffer for near infra red fluorescent Western blotting at room temp for 1 h. Primary antibody was added at a dilution of 1:1000 in blocking buffer and incubated with the blots overnight at 4°C. Membranes were washed five times for five minutes in PBS with .05% Tween 20 after which the membranes were placed in blocking buffer with secondary antibody IR dye conjugate at a dilution of 1:10,000. Membranes were again washed five times for five minutes in PBS with .05% Tween 20 after which they were rinsed once with PBS. Western blots were read on a Li-cor Odyssey Infrared Imager (Lincoln, NE).

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**Primary tumor real-time quantitative RT-PCR (Q-RT-PCR):** We used Q-RT-PCR to investigate expression of *MCT1/SLC16A1* in 83 primary neuroblastoma samples. One  $\mu\text{g}$  of total RNA was used for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Q-RT-PCR was performed using an ABI Prism SDS-7900HT thermal cycler (Applied Biosystems, Foster City, CA) in two separate reactions with differently labeled *MCT1* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). We obtained RNA-specific primers and probes from ABI "Assay-on Demand" (catalog number Hs00161826m1 for *MCT1*). Relative expression of target genes was determined by normalization to *GAPDH* according to the manufacturer's instructions. All Q-RT-PCR experiments included a no-template control and were performed in triplicate.

**RT-PCR on established cell lines:** Three neuroblastoma cell lines, IMR32, Sk-N-SH, and NGP, were cultured at pH 6.5. Extracellular pH was adjusted by raising the  $\text{CO}_2$  to 17% for 48 h, then cells were harvested and total RNA was extracted by using Tri Reagent according to the manufacturer's instructions. Subsequently, RT-PCR was performed. In brief, reverse transcription was carried out using the cloned AMV cDNA Synthesis Kit (Invitrogen; Carlsbad, CA) with random hexamers (25°C for 10 min followed by 50°C for 50 min). For the PCR reaction, Platinum Taq polymerase was used. The sequences of specific primers were as follows:

MCT-1 sense, 5'-TTTCTTTGCGGCTTCCGTTGTTG-3';

MCT-1 antisense, 5'-TCAATTTACCCTTCAGCCCCATGG-3';

MCT-4 sense, 5'-TTTTGCTGCTGGGCAACTTCTTCTG-3';

MCT-4 antisense, 5'-TCACGTTGTCTCGAAGCATGGGTTT-3';

MYCN sense, 5'-CACAAGGCCCTCAGTACCTC-3';

MYCN antisense, 5'-GATCAGCTCGCTGGACTGAG-3';

$\beta$ -actin sense, 5'-TGCGTGACATTAAGGAGAAG-3',



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$\beta$ -actin antisense, 5'-CTGCATCCTGTCGGCAATG-3'.

The cycling parameters are as follows: for MCT1 and MCT4, 35 PCR cycles of 30 s for denaturing at 94°C, 30 sec for annealing at 62°C, and 45 s for DNA synthesis at 72°C; for MYCN, 40 PCR cycles of 30 sec for denaturing at 95°C, 30 s for annealing at 55°C, and 1 min for DNA synthesis at 72°C; for  $\beta$ -actin, 25 PCR cycles of 15 s for denaturing at 94°C, 30 s for annealing at 55°C, and 1 min for DNA synthesis at 68°C. PCR products then underwent electrophoresis on ethidium bromide-stained 2% agarose gels.

### **Intracellular pH measurements**

**Cell preparation:** Cells were plated on coverslips affixed to microwell dishes at a density of  $0.2 \times 10^6$  cells in 2 ml of medium, 24-48 h prior to experiments. Coverslips were coated prior to plating with a 1:3 dilution of Matrigel in serum-free medium as previously described (Wahl et al., 2000). Preliminary experiments (not shown) demonstrated that dye leakage during an experiment of 1 h or less was undetectable under these conditions.

**Dye Loading:** Cells were incubated for 15 min with 9  $\mu$ M C.SNARF-1AM (Owen et al., 1997) in medium containing 10% FBS in a 37°C incubator under 5% CO<sub>2</sub> as previously described (Wahl et al., 1997; Wahl et al., 2000). Following a change of medium, we further incubated the cells were further incubated for 20 min at 37°C, 5% CO<sub>2</sub>, to complete hydrolysis of the dye ester to C.SNARF. Each plate was then mounted on the microscope stage and warmed to 37°C under flowing humidified air containing 5% CO<sub>2</sub>. In experiments in which extracellular pH was lowered, this was done by lowering the bicarbonate concentration in the medium while keeping the sodium concentration constant.

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**Fluorescence microscopy and calibration:** Intracellular pH values were obtained from intracellular whole emission spectra of the pH-sensitive dye C.SNARF-1 (cleaved from the membrane permeable AM form of the dye), The dye was loaded into cells before mounting them on an inverted fluorescence microscope. The whole-spectrum approach to measurement, calibration technique, and experimental methodology have been described in detail (Wahl et al., 2002).

**Protocol for extended intracellular pH time courses:** The experiments were performed on substrate-adherent cells at 37°C in complete growth medium. Initially, steady-state for intracellular pH were determined of cells in 2 ml of complete growth medium. The initial steady-state intracellular pH at extracellular 7.3 was measured several times on a field of 8-15 cells. We then replaced the medium with medium containing lonidamine and/or having an extracellular pH of 6.5. We then measured the intracellular pH for 30 min, during which it equilibrated. The fluorescence signal used to measure intracellular pH was collected on a single field of cells for 5 sec of every 10 sec during the course of experiments.

**[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) assay:** The MTS assay (Sladowski et al., 1993) was performed to determine effects of lonidamine on metabolic viability under conditions designed to match the experiments in which intracellular pH measurements were made. Cells were seeded in 96-well culture plates (10,000 cells/well) and after an overnight pre-incubation, cells were exposed to varying concentrations of lonidamine for 48 h. The MTS reagents were then mixed and added to quantitate in a colorimetric assay the number of viable cells present in each treatment group. Color development was

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monitored with a spectrophotometer at 490 nm. The effect of lonidamine on metabolic viability was expressed as the percentage of cells that were viable relative to the percentage of such cells in untreated controls.

**Cell Death Assay:**

Roche Cell Death Detection ELISA Plus 10x was carried out according to the manufacturers suggested methods for measuring both apoptotic cell death and necrotic cell death in adherent cells.

**Statistical Analysis.** Since distributions for gene-expression data are skewed, rametric analyses were used. For the RT-PCR data and clinical correlations, the two-sample Wilcoxon rank-sum test was used to explore possible associations between MCT1 gene expression and age (dichotomized at 1 year of age), stage (4 versus other), risk group (high-risk versus other), Shimada histopathology (unfavorable versus favorable), DNA index (diploid versus other), and MYCN status (amplified versus not amplified). The Kruskal-Wallis test was used to examine gene expression with risk group of three categories. Intracellular pH and MTS data are expressed as means  $\pm$  SE. Student's t-test was used to determine the significance between each experimental group. Box-plots were used to describe gene expression by clinical and biological factors.

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## Results

***MCT1/SLC16A1* mRNA is differentially expressed in neuroblastoma.** Table 1 shows the distribution of clinical and biological co-variables for the 83 neuroblastomas studied. Q-RT-PCR first showed high concordance with Affymetrix microarray information (Wang et al., 2006). High *MCT1/SLC16A1* mRNA expression was significantly associated with age > one year at diagnosis, stage 4 disease, unfavorable Shimada histopathology diploid DNA index, and *MYCN* amplification, and high-risk clinical group by COG criteria as shown in Figure 1.

**Neuroblastoma cell lines exhibit *MCT1* message.** Analysis of cell lines by RT-PCR at normal and low pH showed that *MYCN* and *MCT1* were expressed in the IMR32 and NGP cell lines, both of which have *MYCN* amplification (Fig. 2). In contrast, Sk-N-SH cells with no genomic amplification of the *MYCN* locus, expressed low amounts of *MCT1* and little *MYCN*. In NGP cells, *MYCN* and *MCT1* were high at both normal and low extracellular pH. In all cases, *MYCN* expression and *MCT1* expression were positively correlated.

**Neuroblastoma cells lines exhibit MCT protein.** Analysis of cell lines by Western blot indicate that MCT is expressed in all of the cell lines tested. Figure 3 shows similar expression of this protein in IMR32, NGP, and Sk-N-SH cells.

**Lonidamine dose response during acute acidification of CHP134 cells.** Figure 4 shows the effect of lonidamine, an MCT inhibitor, on the intracellular pH of cells having an extracellular pH of 6.5. Lonidamine immediately exerted a lowering effect on intracellular pH, which reached a minimum level within 15-30 min. On continued exposure to lonidamine, a new steady-state intracellular pH was sustained. In this cell line at the highest dose of lonidamine the intracellular pH decreased to below 6.5.

**Lonidamine lowered intracellular pH at low extracellular pH.** After extracellular acidification, intracellular pH was measured in IMR32, Sk-N-SH, and NGP

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cells after extracellular acidification combined with various concentrations of lonidamine to demonstrate a dose response (Table 2). All three cell lines showed concentration-dependent accentuation of the intracellular acidification achieved by lowering extracellular pH alone. However, the MYCN-amplified cell lines IMR32 and NGP showed more intracellular acidification. Similar results were obtained by incubating cells with increasing concentrations of extracellular sodium lactate at concentrations of 40, 80, and 160 mM (Table 2).

Lonidamine had no effect on intracellular pH when the extracellular pH was maintained at pH 7.3 (data not shown). When the experiments were repeated at an extracellular pH of 6.5, the intracellular pH of lonidamine-treated cells decreased significantly relative to the values achieved with extracellular acidification alone. For SKNSH cells, acute acidification to 6.5 caused intracellular pH to decrease from  $7.41 \pm 0.003$  to  $7.25 \pm 0.003$ , a decrease of 0.16 unit. When lonidamine was added at 160, 240, or 320  $\mu\text{M}$ , the net decreases in pH when combined with acidification were 0.21, 0.24, and 0.31 pH unit. For NGP cells, acidification alone decreased intracellular pH from  $7.30 \pm 0.07$  to  $7.01 \pm 0.06$ , an average decrease of 0.23 unit. When lonidamine was added at 160, 240, or 320  $\mu\text{M}$ , the net decreases in pH were 0.34, 0.52, and 0.55, respectively. For IMR32 cells, the initial intracellular pH of  $7.47 \pm 0.07$  was decreased to  $7.03 \pm 0.05$  by acidification to 6.5 giving an average decrease of 0.44 unit. When lonidamine was added at 160, 240, or 320  $\mu\text{M}$ , the net decreases in pH were 0.83, 0.93, and 1.24 units, respectively.

Comparing the results from these cell lines, we found that Sk-N-SH cells, which expressed negligible MCT1 (Fig. 3), showed the least decrease in intracellular pH after lonidamine treatment. IMR32 and NGP cells, which express higher levels of MCT1 (see

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Fig. 2), exhibited much greater decreases in intracellular pH under the same conditions, thus correlating the level of MCT1 expression with the lonidamine response.

**Cell line metabolic activity was compromised by acidification and lonidamine administration.** As shown in Fig. 5, lonidamine had a concentration-dependent cytotoxic effect in all cell lines studied. Compared with treatment at an extracellular pH of 7.3, lowering the extracellular pH to 6.5 induced a significant decrease in metabolic activity with increasing lonidamine concentration.

The results of using increasing concentrations of extracellular lactate or lonidamine in separate experiments measuring intracellular pH are shown in Table 2. When cells were incubated with 10-160 mM lactate, the effects on intracellular pH were virtually identical to those of lonidamine, as well as to those of  $\alpha$ -cyano-4-OH-cinnamic acid (CNCn), an established MCT inhibitor (Dimmer et al., 2000) which has been shown to produce this effect *in vitro* in human melanoma cells (Wahl et al., 2002). These results support the hypothesis that the effect of lonidamine on intracellular pH is to reverse the gradient of lactic acid so that it becomes inwardly directed, causing an immediate and precipitous decline in intracellular pH, followed by decreased metabolic viability.

To determine whether the sudden decline in intracellular pH subsequent to lonidamine treatment was sufficient to cause cell death the Cell Death Detection ELISA was performed. Figure 7 shows that both IMR32 and Sk-N-SH cells undergo apoptotic death in a dose dependent fashion when subjected to lonidamine treatment at pH 6.7. An increase in absorbance at 405 nm corresponds to an increase in the concentration of nucleosomes, complexes of fragments of DNA and histones, in the cytoplasm of cells lysed in the apoptosis assay. IMR32 neuroblastoma cells showed a three-fold increase in nucleosome concentration with 160  $\mu$ M lonidamine treatment at pH 6.7, Sk-N-SH neuroblastoma cells showed a four-fold increase in nucleosome concentration with 160

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$\mu$ M lonidamine treatment at pH 6.7. Enrichment in nucleosomes in the cytoplasm of cells is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown occurs during apoptosis. Very little or no increase in cell death resulted from lonidamine treatment at pH 7.3. Lonidamine treatment at either pH had no effect on necrotic cell death.

Figure 8 shows the effects of acute acidification plus lonidamine on neuroblastoma cells lacking mitochondria. In these rho-negative cells, an intracellular pH increase follows the acute extracellular pH decrease from 7.3 to 6.5. This increase was sustained during the time course of treatment. The dose response to lonidamine indicates that these cells, in spite of their lack of mitochondria, are responsive to lonidamine. This is not surprising, given that cells without mitochondria, would, in any case, produce fewer metabolic protons than do cells with mitochondria. When lonidamine was administered, the intracellular pH in these cells decreased as a function of time in a manner similar to that in cells with mitochondria.

## Discussion

We have demonstrated, using primary biopsies and cell lines, that *MCT1* activity is highly associated with *MYCN* amplification in human neuroblastoma. Thus, as is the case with *MYCN*, *MCT1* expression appears to correlate with high-risk disease features such as the presence of metastatic disease at diagnosis. However, *MYCN* is not likely to be a good therapeutic target since it is a transcription factor affecting the activity of numerous proteins (Dang et al., 1999). This work supports the hypothesis that *MCT1* may be a more useful therapeutic target. The functional assays of *MCT* activity show that inducibility of the gene at low pH or amount of protein by Western blot are not predictive of how much an *MCT* inhibitor can affect intracellular pH. The findings indicate that inducibility as a function of extracellular pH was variable. The finding that the *MCT1*

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can be constitutively on or off or pH inducible is indeed a surprising finding. We think this may be indicative of variations in regulation of this gene. It may indirectly be affected by the relative amounts of oxidative aerobic metabolism vs. anaerobic metabolism that varies within a tumor in space and time. It may also be due to the deregulation of normal signal transduction pathways, common to many forms of cancer. Our experiments in which we attempted to knock-down MYCN and look at effects on MCT1 were inconclusive, as were similar experiments performed on MYCN by Tang et al (Tang et al., 2006).

Treating neuroblastoma cells with the MCT inhibitor lonidamine induced a dramatic and immediate decrease in intracellular pH (Fig. 3). This correlated with a significant loss of viability (Fig. 4) when extracellular pH decreased to 6.5, which is similar to the acidic microenvironmental pH of most tumors. MCT inhibitors can thus take advantage of the acidic pH tumor microenvironment to confer selectivity.

Our studies suggest that the mechanism by which sustained low intracellular pH can bring about cell death is primarily apoptosis. Watching closely as the cell medium was acidified and test compounds were added, we observed not only that the SNARF fluorescence per cell decreased 75% within the first 15 m of treatment, but also that the cells did not detach and float. Dye leakage is an indication of compromised membrane integrity and viability, and impending karyolysis. We saw no signs of this from acute extracellular acidification without drug nor did extracellular acidification without drug cause any significant increase in apoptosis or necrosis by cell death ELISA.

The declines in intracellular pH to below 6.5 are significant in that intracellular metabolism ceases below 6.5; acidic endonucleases are activated and apoptosis can ensue (Eastman, 1995). Also, all cellular enzymes have pH optima for their activity, such that effects on the myriad of cytosolic and mitochondrial enzymes within the cells are certain to be multiple. This has led to considerable confusion in the literature



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regarding the identity of the lonidamine receptor. Another effect of lowering intracellular pH is the modification of protein folding in membranes, causing alterations in ligand binding to membrane-bound proteins.

Members of the MCT family of transporters are responsible for the export from cells of lactate, pyruvate, and a variety of other monocarboxylates (Dimmer et al., 2000). They are also referred to as organic anion symporters because they transport monocarboxylates in the same direction as protons. Consequently, we predicted that inhibition of these transporters could alter intracellular pH regulation, resulting in an inability of tumor cells to live in their acidic microenvironment.

To date, 14 MCT isoforms with various substrate specificities have been identified (Halestrap and Meredith, 2004). MCT1 is ubiquitous, with its highest levels found in skeletal muscle (Kim-Garcia et al., 1995). In most tissues, MCT1 is primarily a lactic acid transporter. The present study demonstrates that although MCT exchangers export lactic acid rather than regulate intracellular pH, inhibition of their activities can dramatically affect intracellular pH.

MCT1 gene expression has been reported in a few tumor cell lines other than neuroblastoma or melanoma (Lin et al., 1998), but MCT4 levels had not been measured in any tumors before our demonstration of elevated MCT1 and MCT4 activity in human melanoma cells grown at low extracellular pH (Wahl et al., 2002). The present study provides the first demonstration that the MCT transporters can be critical for maintaining intracellular pH in the viable range in neuroblastoma. Our results indicate that inhibitors of MCT could be useful adjuncts to neuroblastoma therapy, having particularly high activity where extracellular pH is low. Selectivity for the tumor microenvironment is based on the known low pH properties of the tumor. It has recently been reported that siRNA silencing of MCT in malignant glioma cells resulted in a precipitous decline in intracellular pH and cell death (Mathupala et al., 2004)

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Lonidamine was tested as an anti-cancer drug in the 1980s and 1990s before much information was available regarding its mechanism of action. It was first reported that lonidamine inhibits lactate production in malignant gliomas but not in normal tissue, although the molecular mechanism for the lactate export was unknown (Paggi et al., 1988). Based on subsequent studies using magnetic resonance spectroscopy, lonidamine was proposed to be an inhibitor of lactic acid efflux that causes intracellular acidification (Ben-Horin et al., 1995)). Increases in intracellular acidification in brain tumors were demonstrated. The present study provides *in-vitro* support for these conclusions and extends the observation that inhibition of lactate efflux might be a therapeutically beneficial means of bringing about acidification to treat neuroblastoma.

Based on evidence from our own work and that of others (Ben-Horin et al., 1995; Ben-Yoseph et al., 1998; Floridi et al., 1981; Mardor et al., 2000; Pratesi et al., 1996; Pulselli et al., 1996; Stryker and Gerweck, 1988; Zhou et al., 2001), we propose that lonidamine is an inhibitor of MCT isoforms 1 and 4. Clinical studies testing the anti-tumor activity of lonidamine in adults found that its intravenous administration caused mild muscular and testicular discomfort (Scarantino et al., 1991), the tissues in which MCT1 and MCT4 isoforms are most prevalent (Dimmer et al., 2000; Wilson et al., 1998). When lonidamine was administered orally, however, there were no significant side effects, and an oral administration schedule is currently being studied in benign prostatic hyperplasia.

The present study, with its measurement of the immediate effect on intracellular pH, provides direct evidence of the entry of lactic acid into cells and inhibition of its export from cells subsequent to lonidamine treatment. Floridi et al. (1981) stated that micromolar amounts of lonidamine have major effects on plasma membranes. Our results support this statement, the results shown in Figures 5 and 6 strongly suggest that lonidamine-induced cytotoxicity is at least partly dependent on its inhibition of plasma membrane MCT1. The results shown in Figure 7 show that the mode of cell

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death occurring during lonidamine exposure in IMR32 cells and Sk-N-SH cells is apoptosis as opposed to necrosis. Furthermore, as shown in Figure 8, extracellular acidification of rho-negative (mitochondria-deficient) cells caused slight intracellular alkalinization due to the lack of proton production. These cells nevertheless exhibited dose-dependent acidification in response to increasing concentrations of lonidamine in an acidic extracellular environment. Many types of tumors have high levels of lactate dehydrogenase (Koslowski et al., 2002; Kushner et al., 2003) and lactic acid (Brizel et al., 2001; Walenta et al., 1997). Recently, MCT1 and MCT4 were found to be active at low pH in human melanoma cells (Wahl et al., 2002). No ion gradients are involved other than the gradients of lactate anions and the protons themselves for MCT activity. In a tumor with an acidic milieu, a lactate gradient could be used to drive H<sup>+</sup> transport against its transmembrane gradient, as originally described by Warburg (Warburg, 1930). The combined lactate and proton gradients provide the only requirement for transport into or out of the cell, since each requires the other to have electroneutral movement through the plasma membrane.

In conclusion, the results presented here indicate that MCT activity in neuroblastoma is high and correlates with high amplification of *MYCN*. Inhibitors of MCT have great potential when used to achieve selective compromise of neuroblastoma either by directly interfering with the viability of cells or improving the effectiveness of chemotherapeutic agents that function best at low extracellular pH, such as alkylating agents and platinum-containing compounds, while sparing normal and surrounding cells at normal extracellular pH. Clearly, further exploration is needed into what other tumor and cell types express high levels of MCT1 on their cell surface and more importantly how the inhibition of MCT affects their cellular homeostasis. Additionally, other considerations that pertain *in vivo* are the level of hypoxia and perfusion, which the present study did not address. Our study does indicate that extracellular pH can be a critical parameter of

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the tumor microenvironment for use in assessing the potential effectiveness of therapeutic anti-tumor agents.

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**Acknowledgments.** The authors thank Angela M. Spicer, Richel Y. Strydom, and Zhaoxi Zhong for expert technical assistance and the Children's Oncology Group for providing tissue samples. The authors also thank Mark W. Dewhirst, Charles S. Owen, Judith A. Owen, Salvatore V. Pizzo, Jeanne Cole and Pierre Sonveaux for expert editorial assistance.

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**Footnotes:** Grant support: National Institutes of Health Grant P01 CA-56690 (M.L. Wahl), R01CA87847 (J.M. Maris), The Abramson Family Cancer Research Institute (J.M. Maris), The Children's Oncology Group (J.M. Maris) and an American Cancer Society Fellowship (M.J. Morowitz), and National Institutes of Health Medical Scientist Training Program T32GM07171 (Q.J. Quinones).

## Figure Legends:

### **Fig. 1 Correlation of MCT1 mRNA expression with stage classification in**

**neuroblastoma.** The categories that the NB biopsies were divided into are as follows:

Human Fetal (H.F.) Brain (normal), Low Risk, Intermediate (Int.) Risk, High Risk, and

Ultra High Risk NB Groups. These clinical categories are further defined as follows:

Low risk NB were completely resectable, with no metastases. Moderate Risk were *not* completely resectable (incomplete gross excision), with no metastases. High Risk were those with ipsilateral lymph node involvement. Ultra High Risk were those with widely disseminated disease (commonly to liver, bone marrow, and/or skin, and other organs).

### **Fig. 2 Expression of MCT1, MCT4 and MYCN mRNA in three neuroblastoma cell**

**lines by RT-PCR.** Cells were cultured at pH 7.3 or 6.5 for 48 h, after which the cells were harvested and their total RNA extracted for RT-PCR. Lane 1, IMR32 cells at pH 7.3; lane 2, IMR32 cells at pH 6.5; lane 3, Sk-N-SH cells at pH 7.3; lane 4, Sk-N-SH cells at pH 6.5; lane 5, NGP cells at pH 7.3; lane 6, NGP cells at pH 6.5. The control in all lanes was  $\beta$ -actin. This experiment was repeated with similar results.

### **Fig. 3 A-B Expression of MCT protein in three neuroblastoma cell lines.**

Cells were cultured at pH 6.5 or 7.3 for 48 h, after which the cells were harvested and their protein was extracted for Western blot. Lane 1: Molecular weight standards, Lane 2, Sk-N-SH cells at pH 6.5; lane 3, Sk-N-SH cells at pH 7.3; lane 4, NGP cells at pH 6.5; lane 5, NGP cells at pH 7.3; lane 6, IMR32 cells at pH 6.5; lane 7, IMR32 cells at pH 7.3. The western blot with anti-MCT1 is shown in part A. The control in all lanes was  $\beta$ -actin shown in part B. This experiment was repeated with similar results.

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**Fig. 4 Intracellular pH dose response to lonidamine with and without acute acidification in CHP134 cells.** Cultured cells were subjected to increasing doses of lonidamine at pH 6.5. Cell viability was measured using the MTS assay. A dose response was observed from 80-320  $\mu$ M. Intracellular pH was lowered to 6.5 or lower from 160-320  $\mu$ M. Filled squares: untreated. Open squares: acute acidification to 6.5. Filled diamonds: acute acidification plus DMSO vehicle. Open diamonds: 80  $\mu$ M lonidamine plus acute acidification. Filled triangles: 160  $\mu$ M lonidamine plus acute acidification. Open triangles: 240  $\mu$ M lonidamine plus acute acidification. Small filled squares: 320  $\mu$ M lonidamine plus acute acidification.

**Fig. 5A-C Cell viability as a function of lonidamine concentration.** A. IMR32 B. Sk-N-SH C. NGP Cultured cells were subjected to increasing doses of lonidamine at pH 7.3 and 6.5 for 48 h. Viability was measured using the MTS assay. A dose response was observed from 160-320  $\mu$ M. The percentage of IMR32 cells that died during exposure to lonidamine at pH 6.5 was  $23.9 \pm 6.3\%$ ; the percentage of Sk-N-SH cells that died under the same conditions was  $38.3 \pm 3.0\%$ . [The following information should be shifted to the legend for Fig. 4. (Fig. 4A,B). Among NGP cells,  $3.1 \pm 2.4\%$  were viable when exposed to 160  $\mu$ M lonidamine at pH 6.5 (Fig. 4C). Data are means  $\pm$  SE; \*,  $P < 0.01$  ( $n = 8$ ).]

**Fig. 6A-C Cell viability as a function of lactate concentration.** A. IMR32 B. Sk-N-SH C. NGP Cultured cells were subjected to increasing doses of lactate at pH 7.3 and pH 6.5. Viability was measured using the MTS assay. A dose response was observed from 10-80 mM. In all cell lines, there was a statistically significant difference between results obtained at pH 7.3 and pH 6.5; this was most pronounced in IMR32 cells (Fig. 4A). Data are means  $\pm$  SE; \*,  $P < 0.01$  ( $n = 8$ ).

**Fig. 7 Apoptosis assay of three neuroblastoma cell lines exposed to lonidamine and acidification.** A cell death detection assay was performed by ELISA that allows for

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determination of whether cytoplasmic histone-associated DNA fragmentation occurs after induction of cell death. This assay distinguishes apoptosis from necrosis. Cells were cultured at pH 6.7 or 7.3 and treated with vehicle or lonidamine for 24 hrs. Solid bars represent results in experiments performed at pH 6.7. Open bars represent experiments performed at pH 7.3. An increase in absorbance at 405 is indicative of increased cell death. Panel A is IMR-32 cells and Panel B is Sk-N-SH.

**Fig. 8 Response of rho-negative Sy5Y cells to lonidamine when coupled with acute acidification.** A steady-state intracellular pH was obtained, followed by acute extracellular acidification from pH 7.3 to pH 6.5 with or without lonidamine in a dose response. Diamonds: Vehicle plus acute acidification. Squares: 120  $\mu$ M lonidamine with acute acidification. Triangles: 240  $\mu$ M lonidamine with acute acidification. Circles: 320  $\mu$ M lonidamine with acute acidification.

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**Table 1 Summary of Taqman PCR (MCT1/GAPDH ×10)**

Variables	N	Median (Min, Max)	P-value
Age at Diagnosis			.033
<1 year	36	5.89 (3.24, 10.5)	
>1 year	47	7.25 (2.02, 14.4)	
Stage			.0028
1, 2	24	5.30 (2.02, 9.55)	
3, 4	59	7.10 (2.17, 14.4)	
Stage			.0009
1,2,3	43	5.65 (2.02, 10.1)	
4	40	7.63 (2.87, 14.4)	
Risk Group			.0016
Low	24	5.30 (2.02, 9.55)	
Intermediate	19	6.55 (2.17, 10.1)	
High and Ultrahigh	40	7.96 (2.87, 14.4)	
Risk Group			.0012
Low and Intermediate	43	5.87 (2.02, 10.1)	
High and Ultrahigh	40	7.96 (2.87, 14.4)	
Shimada			.0032
Favorable	42	5.89 (2.02, 10.1)	
Unfavorable	39	7.76 (2.87, 14.4)	
DNA Index			.028
Diploid	21	7.34 (2.87, 14.4)	
Hyperploid	57	6.05 (2.02, 12.4)	
MYCN			<.0001
Amplification	16	9.46 (4.73, 14.4)	
Single Copy	67	5.91 (2.02, 10.1)	

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**Table 2 Acidification with and without MCT inhibitors in neuroblastoma cells**

<b>Extracellular pH before and after drug addition</b>	<b>NGP cells Intracellular pH</b>	<b>Sk-N-SH cells Intracellular pH</b>	<b>IMR-32 cells Intracellular pH</b>
7.3→7.3	7.30±0.01	7.41±0.01	7.47±0.01
7.3→6.5+vehicle	7.07±0.06	7.25±0.003	7.03±0.02
7.3→6.5+ 160 µM lonidamine	6.96±0.02	7.20±0.02	6.64±0.01
7.3→6.5+ 240 µM lonidamine	6.78±0.03	7.17±0.04	6.54±0.01
7.3→6.5+ 320 µM lonidamine	6.75±0.02	7.10±0.02	6.23±0.24
7.3→6.5+40 mM NaLactate	7.04±0.02	7.22±0.02	6.68±0.06
7.3→6.5+80 mM NaLactate	6.95±0.02	7.13±0.02	< 6.0
7.3→6.5+160 mM NaLactate	6.88±0.02	7.08±0.02	< 6.0
7.3→6.5+1.25 mM CNCn			< 6.0
7.3→6.5+2.5 mM CNCn			< 6.0
7.3→6.5+5 mM CNCn			< 6.0

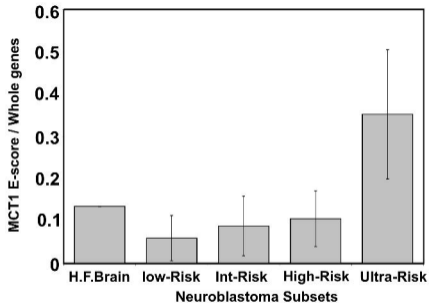


Figure 01

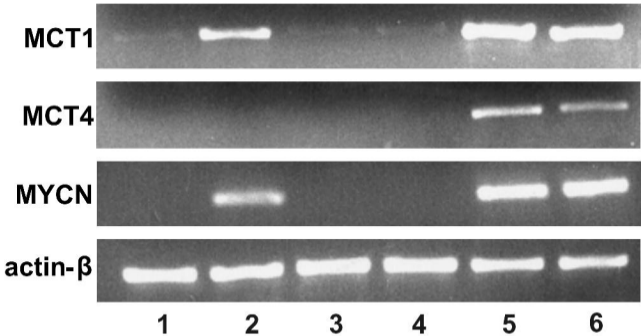
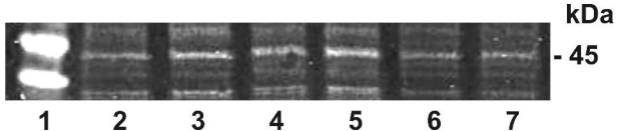


Figure 02





**Figure 03**

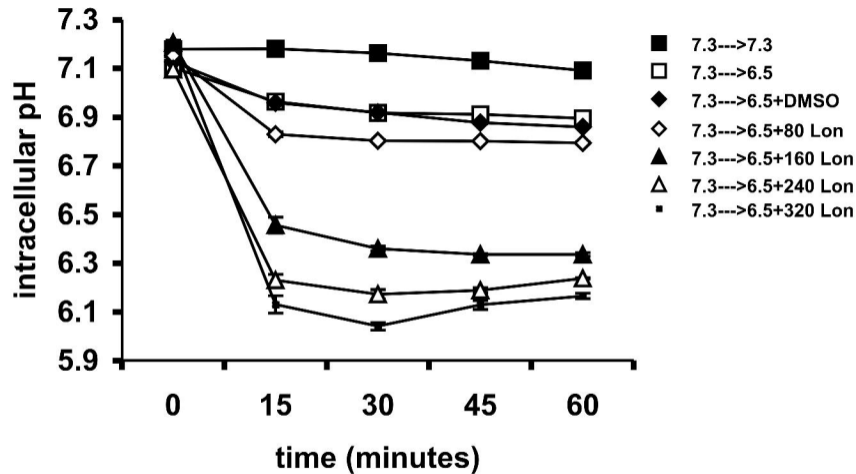


Figure 04

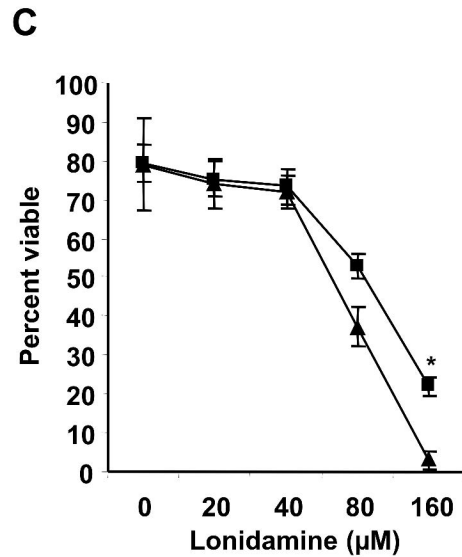
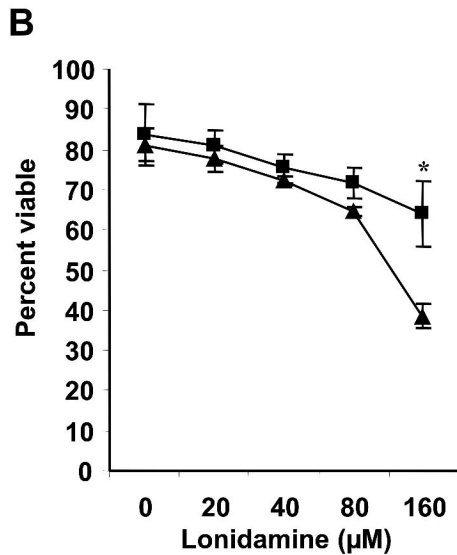
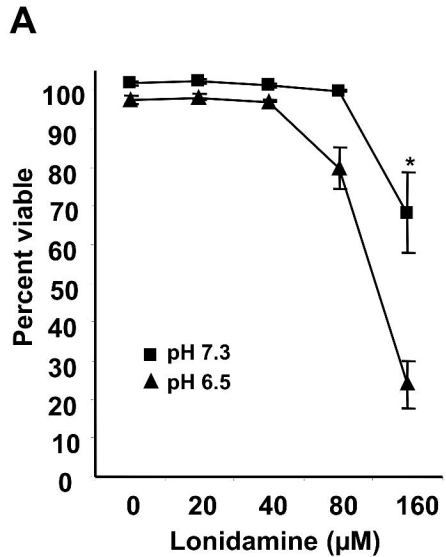


Figure 05

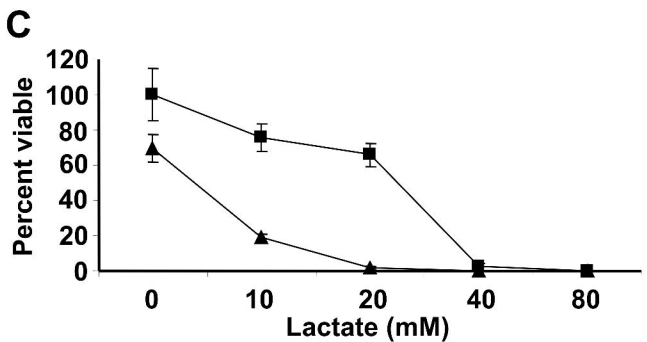
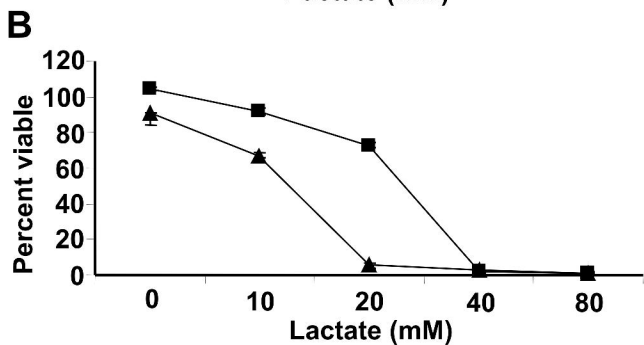
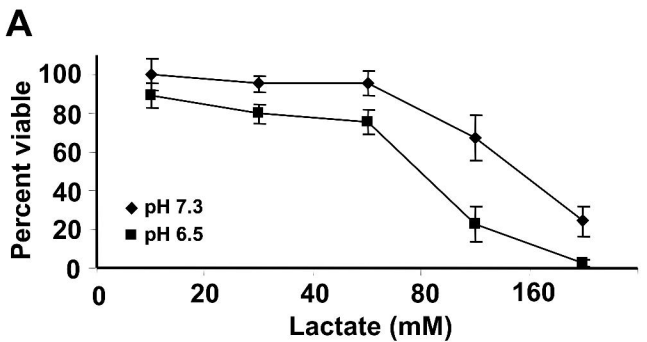


Figure 06

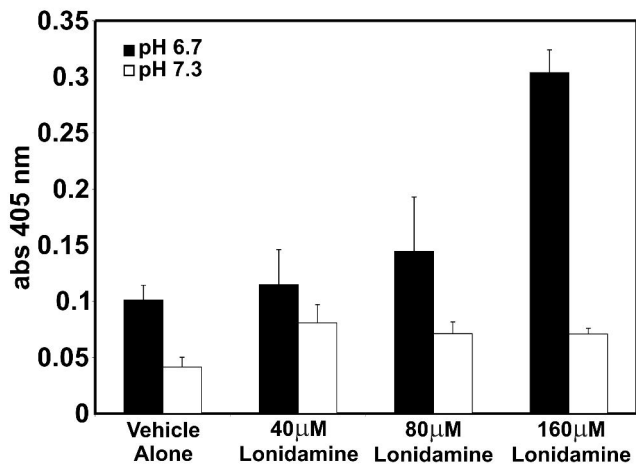
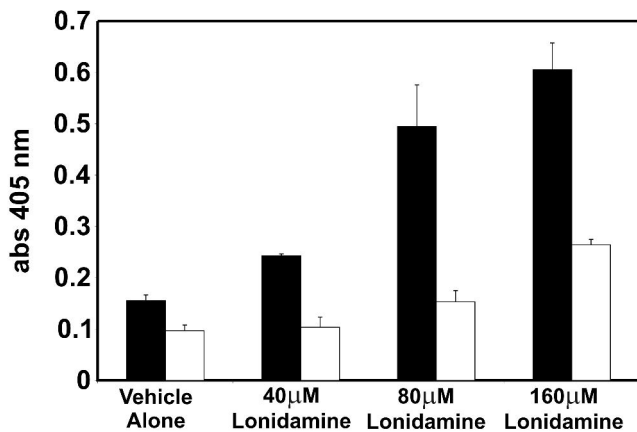
**A****B**

Figure 07

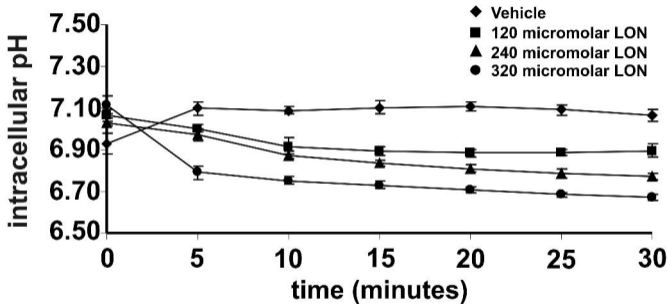


Figure 08